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2
3 **Displacement Assay for Selective Biological**

4 **Material Detection**

5
6 **Reference to Related Applications**

7 This application seeks benefit of the filing date of
8 U.S. Provisional Application 60/443,299, filed January 28,
9 2003, the contents of which is herein incorporated by
10 reference in its entirety.

11
12 **Field of the Invention**

13 This invention relates to the detection of pathogenic
14 microorganisms, or biological materials, and more
15 particularly relates to a composite bioassay material useful
16 for the detection of particular toxic substances, its method
17 of manufacture and method of use, wherein the composite
18 material is particularly useful for food packaging and the
19 like, and is capable of simultaneously detecting and
20 identifying a multiplicity of such biological materials.

21
22 **Background of the Invention**

23 Although considerable effort and expense have been put
24 forth in an effort to control food borne pathogenic

1 microorganisms, there nevertheless exist significant safety
2 problems in the supply of packaged food. For example,
3 numerous outbreaks of food poisoning brought about by
4 foodstuffs contaminated with strains of the E-Coli,
5 Campylobacter, Listeria, Cyclospora and Salmonella
6 microorganisms have caused illness and even death, not to
7 mention a tremendous loss of revenue for food producers.
8 These and other microorganisms can inadvertently taint food,
9 even when reasonably careful food handling procedures are
10 followed. The possibility of accidental contamination, for
11 example by temperature abuse, in and of itself, is enough to
12 warrant incorporation of safe and effective biological
13 material diagnosis and detection procedures. Further
14 complicating the situation is the very real possibility that
15 a terrorist organization might target either the food or
16 water supply of a municipality or even a nation itself, by
17 attempting to include a pathogenic microorganism or toxic
18 contaminant capable of causing widespread illness or even
19 death. If, by accident or design, the food supply of a
20 particular population were to be contaminated, it is not only
21 imperative that the population be alerted to the
22 contamination, but it is further necessary that the
23 particular contaminant be quickly and precisely pinpointed so
24 that appropriate countermeasures may be taken.

1 Thus, if it were possible to readily substitute standard
2 packaging materials with a flexible material capable of
3 1) quickly and easily detecting the presence, and
4 2) indicating the particular identity of a variety of
5 pathogenic biological materials, a long felt need would be
6 satisfied.

7

8 Description of the Prior Art

9 The Berkeley Lab Research News of 12/10/96, in an
10 article entitle "New Sensor Provides First Instant Test for
11 Toxic E.Coli Organism" reports on the work of Stevens and
12 Cheng to develop sensors capable of detecting E. Coli strain
13 0157:H7. A color change from blue to red instantaneously
14 signals the presence of the virulent E. Coli 0157:H7
15 microorganism. Prior art required test sampling and a 24
16 hour culture period in order to determine the presence of the
17 E. Coli microorganism, requiring the use of a variety of
18 diagnostic tools including dyes and microscopes. An
19 alternative technique, involving the use of polymerase chain
20 reaction technology, multiplies the amount of DNA present in
21 a sample until it reaches a detectable level. This test
22 requires several hours before results can be obtained. The
23 Berkeley sensor is inexpensive and may be placed on a variety
24 of materials such as plastic, paper, or glass, e.g. within a

1 bottle cap or container lid. Multiple copies of a single
2 molecule are fabricated into a thin film which has a two part
3 composite structure. The surface binds the biological
4 material while the backbone underlying the surface is the
5 color-changing signaling system.

6 The Berkeley researchers do not teach the concept of
7 incorporating any means for self-detection within food
8 packaging, nor do they contemplate the inclusion of multiple
9 means capable of both detecting and identifying the source of
10 pathogenic contamination to a technically untrained end user,
11 e.g. the food purchaser or consumer.

12 Wang et al, in an article entitled "An immune-capturing
13 and concentrating procedure for Escherichia coli 0157:H7 and
14 its detection by epifluorescence microscopy" published in
15 Food Microbiology, 1998, Vol. 15 discloses the capture of E.
16 coli on a polyvinylchloride sheet coated with polyclonal
17 anti-E. coli 0157:H7 antibody and stained with fluorescein-
18 labeled anti-E. coli 0157:H7. After being scraped from the
19 PVC surface, the cells were subjected to epifluorescence
20 microscopy for determining presence and concentration. The
21 reference fails to teach or suggest the concept of
22 incorporating any means for self-detection within food
23 packaging, nor does it contemplate the inclusion of multiple
24 means capable of both detecting and identifying the source of

1 pathogenic contamination to a technically untrained end user,
2 e.g. the food purchaser or consumer, and especially fails to
3 disclose such detection without the use of specialized
4 detection techniques and equipment.

5 U.S. Patent 5,776,672 discloses a single stranded
6 nucleic acid probe having a base sequence complementary to
7 the gene to be detected which is immobilized onto the surface
8 of an optical fiber and then reacted with the gene sample
9 denatured to a single stranded form. The nucleic acid probe,
10 hybridized with the gene is detected by electrochemical or
11 optical detection methodology. In contrast to the instantly
12 disclosed invention, this reference does not suggest the
13 immobilization of the probe onto a flexible polyvinylchloride
14 or polyolefin film, nor does it suggest the utilization of
15 gelcoats having varying porosities to act as a control or
16 limiting agent with respect to the migration of antibodies or
17 microbial material through the bioassay test material, or to
18 serve as a medium for enhancement of the growth of the
19 microbial material.

20 U.S. Patent 5,756,291 discloses a method of identifying
21 oligomer sequences. The method generates aptamers which are
22 capable of binding to serum factors and all surface
23 molecules. Complexation of the target molecules with a
24 mixture of nucleotides occurs under conditions wherein a

1 complex is formed with the specific binding sequences but not
2 with the other members of the oligonucleotide mixture. The
3 reference fails to suggest the immobilization of the aptamers
4 upon a flexible polyvinylchloride or polyolefin base
5 material, nor does it suggest the use of a protective gelcoat
6 layer which acts as a means to selectively control the
7 migration of antibodies and antigens, or to serve as a medium
8 for enhancement of the growth of microbial material.

9 10 Summary of the Invention

11 The present invention relates to a displacement assay
12 particularly adapted for use in packaging materials for food
13 and other products, along with methods for their manufacture
14 and use. The presence of undesirable biological materials in
15 the packaged material is readily ascertained by the consumer,
16 merchant, regulator, etc. under ordinary conditions and
17 without the use of special equipment. A multiplicity of
18 biological materials threaten our food supply. The present
19 invention provides a unique composite material capable of
20 detecting and identifying multiple biological materials
21 within a single package. The biological material
22 identification system is designed for incorporation into
23 existing types of flexible packaging material such as
24 polyvinylchloride and polyolefin films, and its introduction
25 into the existing packaging infrastructure will require

1 little or no change to present systems or procedures. Thus,
2 the widespread inclusion of the biological material detecting
3 system of the instant invention will be both efficient and
4 economical.

5 In one embodiment of the invention the biological
6 material detecting system prints a pattern containing several
7 antibodies or aptamers, derived from plant or animal origins,
8 onto a packaging material which is usually a type of
9 polymeric film, preferably a polyvinylchloride or polyolefin
10 film and most preferably a polyethylene film which has
11 undergone a surface treatment, e.g. corona discharge to
12 enhance the film's ability to immobilize the antibodies upon
13 its surface. The agents are protected by a special abrasion
14 resistant gel coat in which the porosity is tailored to
15 control the ability of certain antibodies, toxic substances,
16 etc. to migrate therethrough. Each antibody is specific to a
17 particular biological material and is printed having a
18 distinctive icon shape. The detection system may contain any
19 number of antibodies capable of detecting a variety of common
20 toxic food microbes; although any number of microbes may be
21 identified via the inventive concept taught herein, for the
22 purpose of this description, the microbes of interest will be
23 limited to E.Coli, Salmonella, Listeria and Cyclospora.

24 An important feature of the biological material
25 detection system is its all-encompassing presence around and
26 upon the product being packaged. Since the biological

1 material detecting system is designed as an integral part of
2 the packaging material and covers all surfaces as utilized,
3 there is no part of the packaged product which can be exposed
4 to undetected microbes. In the past, the use of single
5 location or *in situ* detectors have left a majority of the
6 area around and upon the packaged product exposed to
7 undetected microbes. This greatly increased the chance that
8 a spoiled or tainted product might be inadvertently consumed
9 before the toxic agent had spread to the location of the *in*
10 *situ* detector. The biological material detection system of
11 the present invention avoids this problem by providing a
12 plurality of individual detectors per unit area which are
13 effective to insure positive detection of any pathogenic
14 microorganisms within the product being tested. In order to
15 be effective a particular degree of sensitivity is required,
16 e.g. the detecting system must be capable of positively
17 identifying one microbial cell in a 25 gram meat sample In
18 a preferred embodiment, four detectors per square inch of
19 packaging material surface have been utilized, and in a most
20 preferred embodiment nine or more detectors per square inch
21 are incorporated upon the film's surface.

22 By use of the biological material detection system of
23 the present invention a packager or processor can
24 independently determine the multiplicity and identity of
25 those biological materials against which the packaged product
26 is to be protected. Although it is envisioned that the large

1 majority of biological material detection treated packaging
2 will be generic to approximately four of the most common
3 microbes, the system will nevertheless allow each user to
4 customize the protection offered to the public.

5 The biological material detecting system will not merely
6 detect the presence of biological materials, it will also
7 identify the particular biological materials located in a
8 packaged product. This unique feature allows for the
9 immediate identification of each particular biological
10 material present since the antibodies are specific to a
11 detector having a definitive icon shape or other identifying
12 characteristic. Although the end use consumer is primarily
13 interested in whether a food product is, or is not,
14 contaminated per se, the ability to detect and identify the
15 particular biological material immediately is of immeasurable
16 value to merchants, processors, regulators and health
17 officials. The ability to immediately identify a toxic
18 material will lead to greatly reduced response times to
19 health threats that might be caused by the biological
20 material and will also enhance the ability for authorities to
21 locate the source of the problem. The biological material
22 detecting system of the present invention exhibits an active
23 shelf life in excess of 1 year under normal operating
24 conditions. This enhances the use of a biological material
25 detection system on products which are intended to be stored
26 for long periods of time. If these products are stored so as

1 to be ready for immediate use in some time of emergency, then
2 it is extremely beneficial to definitely be able to determine
3 the safety of the product at the time that it is to be used.

4 One particularly important feature of the biological
5 material detecting system of the instant invention is its
6 ability to quantitatively sensitize the reagents so as to
7 visually identify only those biological materials which have
8 reached a predetermined concentration or threshold level
9 which is deemed to be harmful to humans.

10 For example, almost all poultry meat contain traces of
11 the salmonella bacteria. In most cases, the salmonella
12 levels have not reached a harmful level of concentration.
13 The biological material detecting reagents are designed to
14 visually report only those instances where the level of
15 concentration of biological materials are deemed harmful by
16 health regulatory bodies.

17 The method of production of the biological material
18 detecting system is designed to be easily incorporated within
19 the packaging infrastructure of existing systems without
20 disruption of the systems or the procedures under which they
21 are operating. The biological material detecting system can
22 be incorporated onto packaging films which are produced by
23 the packager, or those which are supplied by a film
24 manufacturer. The apparatus necessary for applying the
25 biological material detecting system may be easily located at
26 the beginning of any continuous process such as printing or

1 laminating and will operate as an integral part of an
2 existing system.

3 The biological material detecting system of the instant
4 invention represents an entirely new packaging material which
5 is designed to inform the consumer of the presence of certain
6 biological materials or pathogens present in food stuffs or
7 other materials packaged within the detecting system. The
8 system is designed so that the presence of a biological
9 material is presented to the consumer in a distinct,
10 unmistakable manner which is easily visible to the naked eye.

11 Recent outbreaks of E.Coli and other health hazards have
12 presented serious problems to the general population and have
13 raised concerns regarding the safety of the food supply.

14 It is an objective of the present invention to provide a
15 biological material detecting system, in the form of a novel
16 displacement assay technology, for protecting the consumer by
17 detecting and unmistakably presenting to the untrained eye
18 visual icons on the packaging material which signify the
19 presence of a number of pathogens in the food stuff or other
20 materials which are at a level harmful to humans.

21 It is another objective of the instant invention to
22 provide a bioassay material wherein an antigen detecting
23 antibody system is immobilized upon the surface of a flexible
24 polyolefin film.

25 It is still another objective of the instant invention
26 to provide a bioassay material wherein an antigen detecting

1 antibody system is immobilized upon the surface of a flexible
2 polyvinylchloride film.

3 It is a further objective of the invention to provide a
4 biological material detecting system which is so similar in
5 appearance and utilization that its use, in lieu of
6 traditional packaging materials, is not apparent to the food
7 processor or other packagers.

8 A still further objective of the present invention is to
9 provide a biological material detecting system which is cost
10 effective when compared to traditional packaging materials.

11 Other objectives and advantages of this invention will
12 become apparent from the following description taken in
13 conjunction with the accompanying drawings wherein are set
14 forth, by way of illustration and example, certain
15 embodiments of this invention. The drawings constitute a
16 part of this specification and include exemplary embodiments
17 of the present invention and illustrate various objects and
18 features thereof.

19

20 **Brief Description of the Figures**

21 Figure 1 is a graph which demonstrates the displacement of
22 HRP-conjugated antibody from *Pseudomonas* coated on a
23 polystyrene plate by varied amounts of free *Pseudomonas* in
24 solution.

25 Figure 2 is a graph demonstrating displacement of HRP-

1 conjugated antibody from Pseudomonas specific
2 lipopolysaccharide;
3 Figure 3 is a photograph of a working assay
4 Figure 4 is an illustration of a displacement assay.

5

6 **Description of the Preferred Embodiment(s)**

7 Antibody Displacement Assay Format

8 With each assay developed for the displacement assay
9 format, the specific antigen could be comprised of different
10 material. For example, the PSEUDOMONAS assay has an
11 oligosaccharide as the specific antigen, the pesticide assays
12 will each have a different small molecule as the specific
13 antigen and other assays could have lipids, proteins, or
14 other biological/chemical substances as the specific antigen.

15 The differing nature of each specific antigen will pose
16 difficulties with the standardization of the chemistry
17 involved in providing pigment to each assay developed.

18 The component common to all assays to be developed will
19 be the antibody. By using the antibody as the displaceable
20 material, the pigmentation chemistry can be directly
21 transferable between assays.

22 It is thus proposed to print the specific antigen, or a
23 facsimile of the specific antigen, on the plastic film and
24 overprint the pigmented antibody. In this way, the pigmented

1 antibody will be displaced by the contaminating test
2 material; indicating a positive response on the plastic film.
3 In accordance with this invention, a "facsimile antigen" is
4 understood to mean any compound which has a controllable
5 affinity for a particular antibody or immunogenic fragment
6 thereof.

7 The graph in Figure 1 demonstrates the displacement of
8 HRP-conjugated antibody from *Pseudomonas* coated on a
9 polystyrene plate by varied amounts of free *Pseudomonas* in
10 solution.

11

12

13 Process Steps - Proof of Concept

14

15 1. Produce Heat-killed *Pseudomonas*

16

17 2. Coat bottom of an ELISA plate

18

19 3. HRP-Ab binds to *Pseudomonas*

20

21 4. Add free heat-killed *Pseudomonas* in solution

22

23 5. After 1 & 4 hrs unbound *Pseudomonas* is washed away

24

1 6. Add Tetramethy benzidine (TMB)

2

3 7. Color evidenced where Ab present

4 This test evidences a proof of concept in that the
5 ability of antibody to be displaced by unbound antibody in
6 accordance with LeChatelier's principles of equilibrium. The
7 ability of said unbound antigen or facsimile antigen to
8 present changing concentration at the interface of the
9 plate/film permits the unbound antigen to successfully
10 compete for the binding sites occupied by the bound and
11 conjugated antibody. The removal of color evidences this
12 principle in action, thereby resulting in controllable areas
13 of color or transparency being evidenced.

14 The antibody is in water soluble varnish (WSV) and
15 antigen is bound to antibody with color indicator such that
16 color forms with binding; upon exposure, bacteria having
17 higher affinity competes out the color, and the color
18 containing antigen is displaced into food.

19 Thus the displacement test goes from color (in this
20 particular case, a blue color) to clear to show a difference
21 in binding.

22 Now referring to Figure 2, by utilizing antibody as the
23 displaceable component, the conjugation of pigment in all
24 assays is standardized, and the regulatory body required

1 "leaching" tests are standardized, as the material most
2 likely to be transferred to foodstuffs will be, in each
3 assay, the conjugated antibody.

4 The graph in Figure 2 demonstrates the displacement of
5 HRP-conjugated antibody from *Pseudomonas* specific to
6 lipopolysaccharide (LPS) coated on a polystyrene plate by
7 free *Pseudomonas* in solution but not by non-specific bacteria
8 (*Salmonella*) nor by the buffer solution.

9 Figure 3 - Displacement on Film

10 Now referring to Figure 3, *Pseudomonas* LPS was printed
11 in an icon shape in a water based varnish on the XY plotter
12 at a concentration of 1mg/mL (not optimized).

13 The strips of plastic were placed in a 50mL bath of 7-4
14 antibody-HRP conjugate for 1 hour at room temperature.

15 The strips were washed with wash buffer and placed in a
16 50mL bath of either wash buffer (control) or heat-killed
17 *Pseudomonas* solution.

18 The strips were then washed in wash buffer and allowed
19 to dry.

20 TMB was added for 20 minutes at room temperature.

21 The results indicated that the 7-4 antibody-HRP
22 conjugate was displaced by the *Pseudomonas* in solution,
23 thereby again proving the displacement assay principle which
24 is at the heart of the instant invention.

1 Illustrative of films which will function in the present
2 invention is a film containing a structural polymer base
3 having a treated surface and incorporating therein a
4 fluorescing antibody receptor and finally a stabilized gel
5 coat. These films may be untreated polyethylene or
6 polyvinylchloride films which are amenable to antibody
7 immobilization by various mechanisms, e.g. by adsorption. In
8 a particular embodiment, the films may be first cleaned, e.g.
9 by ultrasonication in an appropriate solvent, and
10 subsequently dried. For example the polymer sheet may be
11 exposed to a fifteen minute ultrasonic treatment in a solvent
12 such as methylene chloride, acetone, distilled water, or the
13 like. In some cases, a series of solvent treatments are
14 performed. Subsequently the film is placed in a desiccating
15 device and dried. Alternatively, these films may be created
16 by first exposing the film to an electron discharge treatment
17 at the surface thereof, then printing with a fluorescing
18 antibody receptor. Subsequently, a drying or heating step
19 may be utilized to treat the film to immobilize the receptor.
20 Next, the film is washed to remove un-immobilized receptor;
21 the film is then coated with a gel and finally dried.

22 Additional modifications to polyolefin films may be
23 conducted to create the presence of functional groups, for
24 example a polyethylene sheet may be halogenated by a free

1 radical substitution mechanism, e.g. bromination,
2 chlorosulfonation,, chlorophosphorylation or the like.
3 Furthermore, a halodialkylammonium salt in a sulfuric acid
4 solution may be useful as a halogenating agent when enhanced
5 surface selectivity is desirable.

6 Grafting techniques are also contemplated wherein
7 hydrogen abstraction by transient free radicals or free
8 radical equivalents generated in the vapor or gas phase is
9 conducted. Grafting by various alternative means such as
10 irradiation, various means of surface modification,
11 polyolefin oxidation, acid etching, inclusion of chemical
12 additive compounds to the polymer formulation which have the
13 ability to modify the surface characteristics thereof, or
14 equivalent techniques are all contemplated by this invention.

15 Additionally, the formation of oxygenated surface groups
16 such as hydroxyl, carbonyl and carboxyl groups via a flame
17 treatment surface modification technique is contemplated.

18 Further, functionalization without chain scission by
19 carbene insertion chemistry is also contemplated as a means
20 of polyolefin polymer modification.

21 Illustrative of the types of commercially available
22 films which might be utilized are polyvinylchloride films and
23 a straight polyethylene film with electron discharge
24 treatment marketed under the trademark SCLAIR®. The electron

1 discharge treatment, when utilized, renders the film much
2 more susceptible to immobilization of the antibodies on its
3 surface. Additional films which might be utilized are Nylon
4 66 films, for example DARTEK®, a coextrudable adhesive film
5 such as BYNEL® and a blend of BYNEL® with polyethylene film.

6 All patents and publications mentioned in this
7 specification are indicative of the levels of those skilled
8 in the art to which the invention pertains. All patents and
9 publications are herein incorporated by reference to the same
10 extent as if each individual publication was specifically and
11 individually indicated to be incorporated by reference.

12 It is to be understood that while a certain form of the
13 invention is illustrated, it is not to be limited to the
14 specific form or arrangement herein described and shown. It
15 will be apparent to those skilled in the art that various
16 changes may be made without departing from the scope of the
17 invention and the invention is not to be considered limited
18 to what is shown and described in the specification and any
19 drawings/figures included herein.

20 One skilled in the art will readily appreciate that the
21 present invention is well adapted to carry out the objectives
22 and obtain the ends and advantages mentioned, as well as
23 those inherent therein. The embodiments, methods, procedures
24 and techniques described herein are presently representative

1 of the preferred embodiments, are intended to be exemplary
2 and are not intended as limitations on the scope. Changes
3 therein and other uses will occur to those skilled in the art
4 which are encompassed within the spirit of the invention and
5 are defined by the scope of the appended claims. Although
6 the invention has been described in connection with specific
7 preferred embodiments, it should be understood that the
8 invention as claimed should not be unduly limited to such
9 specific embodiments. Indeed, various modifications of the
10 described modes for carrying out the invention which are
11 obvious to those skilled in the art are intended to be within
12 the scope of the following claims.